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Alteration in nuclear organization is a hallmark of cancer cells. Nuclear organization is likely to be dependent on the arrangement of a non chromatin structure, referred to as the nuclear matrix (NM). An increasing number of key proteins have been identified as NM proteins, however, the role played by their organization in the regulation of nuclear function and cell phenotype remains unknown. Using a model of human mammary epithelial cell (HMEC) morphogenesis and tumorigenesis, I have found that the NM protein NuMA progressively redistributes in the nucleus during morphogenesis, to ultimately form large domains that co-localize with splicing factors. Such changes are not observed in tumor cells cultured in the same conditions. Moreover, alteration of the supramolecular organization of NuMA in cells that have undergone morphogenesis, induces changes in chromatin structure and alterations in cellular phenotype. This demonstrates that the supramolecular organization of NuMA regulates the maintenance of HMEC differentiation. Other NM proteins have been identified as potential tumor suppressors or tumor promoters, using 2-D gel electrophoresis of NM extracts prepared from the mammary cancer progression series HMT-3522. These results suggest that the study of NM proteins offers a new potential for anticancer therapy.

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Abbreviations:

HMEC- human mammary epithelial cell; ECM- extracellular matrix; NM-nuclear matrix, BM-
basement membrane; 3D-three-dimensions, 2D-two-dimension; rBM-reconstituted basement
membrane.

INTRODUCTION.

Alteration in nuclear organization is likely to play a critical role in the expression of tumor phenotype (Nickerson, 1998). Nuclear organization is dependent on a non chromatin structure, referred to as the nuclear matrix (for a review see Nickerson et al., 1995). In order to identify the key components of nuclear organization that may lead to the development of new markers for cancer and the utilization of new targets for anticancer therapy, we ought to already understand the role played by nuclear organization in non tumor cells. Therefore we need to work with models of both morphogenesis and tumorigenesis. The Bissell Laboratory has developed a 3-dimensional (3D) system of cell culture in which normal human mammary epithelial cells (HMECs), embedded in extracellular matrix (ECM) enriched for basement membrane components, undergo tissue-like acinus morphogenesis, and tumor HMECs form tumor-like assemblies in which cells keep proliferating (Petersen et al., 1992; Weaver et al., 1995). Using this system and the HMT-3522 HMECs progression series, the Bissell laboratory has shown that the balance between cell membrane receptors (e.g. adhesion receptors, growth factor receptors) is essential for the expression of the normal behavior of HMECs, and that by altering cell-ECM interactions it is possible to revert the tumor behavior to a phenotypically normal behavior (Weaver et al., 1997; Wang et al., 1998). In addition, these studies have demonstrated that tumor reversion occurs although the revertant cells keep the abnormal genotype, characteristic of the original tumor cells, and that it is accompanied with the reorganization of the internal cell structure (e.g. organization of adhesion plaque proteins and cytoskeleton). It is now well documented that the modulation of cell-ECM interactions induces changes in cytoskeletal organization and modulates biochemical signaling, that ultimately lead to alterations in chromatin structure, transcription factor activity, and gene expression (for a review see Lelièvre and Bissell, 1998). However the way signals are transduced within the nucleus and the role played by nuclear organization in the modulation of nuclear function and cell phenotype remain obscure.

Using the reversion model of HMT-3522 HMECs cultured in 3D, I had obtained preliminary data suggesting that the nuclear matrix protein NuMA, may be a potential mediator of ECM signaling and that its distribution changed depending on the cell phenotype (i.e. normal phenotype vs tumor phenotype). Although the distribution of non chromatin components has been shown to be altered in tumor cells compared to normal cells (Grande et al., 1996), and during the differentiation process (Antoniou et al., 1993), this dynamics of nuclear organization is thought to be only a consequence of changes in gene expression, rather than itself participating in the modulation of the cellular phenotype (Antoniou et al., 1993; Singer and Green, 1997).

During the first year of the research project, I have demonstrated that NuMA is an essential mediator of ECM signaling in HMECs. Its nuclear distribution changes with the different steps of ECM-induced HMEC morphogenesis, including proliferation, growth-arrest, and polarization. More importantly, these studies have demonstrated for the first time that the supramolecular organization of non chromatin structural proteins, like NuMA, is important for the maintenance of HMEC differentiation. On another scale, by analyzing 2-D gels of nuclear matrix proteins, we have identified three potential tumor suppressor-like proteins and three potential tumor promoter-like proteins. These data open a new avenue in the search for novel anticancer therapy.

MATERIALS AND METHODS.

Cell Culture
HMT-3522 HMECs were propagated in 2D cultures in chemically defined medium (Weaver et al., 1997) and growth-arrest was induced by removing epidermal growth factor (EGF) for 48 hours. 3D cultures were prepared by embedding single cells in rBM (Matrigel™, Collaborative Research) or collagen-I matrix (Cellagen™ AC-5, ICN Biochemicals Incorporated) in four-well chamber slides (Nalge Nunc International). These cultures were grown for 5-10 days. Growth-arrest and acinus morphogenesis were routinely observed by days 7-9 for S1 cells.

Antibodies and inhibitors
For Western blots and/or immunostaining, we used antibodies against type IV collagen (clone CIV, Dako), β-catenin (clone 14, Transduction Laboratories), SRm160 splicing factor (clone B1C8, 16), lamin B (clone 101-B7, Matritech Inc.), NuMA (clone 204-41, Matritech Inc., and clone B1C11, a gift from Dr. S. Penman), and polyclonal antibodies against Ki-67 (Novocastra Laboratory), acetylated histone H4 (Upstate Biotechnology Incorporated), and p110Rb (Santa Cruz Biotechnology). For bioperturbation assays, we used antibodies against lamins A/C (clone 636, Novocastra Laboratory) and NuMA (clone 22, Transduction Laboratory), in addition to B1C11 and 101-B7. Trichostatin A (Wako Chemicals) was used as an inhibitor of histone deacetylase (40 nM),

Indirect immunofluorescence
Cells were permeabilized *in situ* (0.5% triton in 100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 5 mM $MgCl_2$ containing 1 mM Pefabloc™, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml trypsin inhibitor type II, and 250 µM NaF), fixed in 2% paraformaldehyde, and immunostained as previously described (Weaver et al., 1997).

Image acquisition, processing, and data analysis
Samples were analyzed using a Bio-Rad MRC 1024 laser scanning confocal microscope attached to a Nikon Diaphot 200 microscope. Fluorescence specificity was ensured by sequential fluorophore excitation. NuMA foci were analyzed using Image Space-3D analysis program (Molecular Probes) and normalized to 3D rBM cluster cell number by highlighting and counting each nucleus using Image Space-measure 2D. The voxel threshold was set at 0.2 µ.

Immunoblot analysis
Total cell extracts (2% SDS in phosphate buffered saline pH 7.4, containing 1 mM Pefabloc™, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml trypsin inhibitor type II, and 250 µM NaF) were prepared *in situ* for 2D cultures, or on acini isolated from 3D cultures by dispase treatment (5,000 U/ml caseinolytic activity, Collaborative Research). Equal amounts of protein were separated and immunoblotted as previously described (Weaver et al., 1997).

In situ NM preparation.
In situ NM preparation was as previously described (He et al., 1990), except that 0.05 % triton and micrococcal nuclease (5 U/ml; Sigma) were used.

Antibody-mediated perturbation of nuclear organization.

rBM induced-acini (day 10) were permeabilized for less than two minutes *in situ* (0.01% digitonin in 25 mM Hepes pH 7.2, 78 mM KHOAc, 3 mM MgHOAc, 1 mM EGTA, 300 mM sucrose, and 1% RIA grade bovine serum albumin), rinsed twice in digitonin-free buffer, and incubated in medium containing dialyzed specific or mock antibodies (15 µg/ml) for 48 hours, after which the cells were incubated with fresh medium for an additional 48 hours. Antibody concentrations and incubation times were determined empirically. Trypan blue dye exclusion tests and apoptosis studies verified the absence of digitonin toxicity.

Preparation of nuclear matrix-associated proteins for 2D gels.

The preparation of nuclear matrix associated proteins (NMPs) was performed according to the protocol in use in Dr. Getzenberg laboratory (Getzenberg et al., 1991). Briefly cells were permeabilized in 0.5 % triton in the presence of a cocktail of protease inhibitors, DNA was degraded using DNAse1, and DNA and soluble proteins were eluted using 0.25 M ammonium sulfate. RNA was removed using RNAse A. Intermediate filament network was disassembled and further reassembled during overnight dialysis. Cytoskeleton components were discarded after ultracentrifugation. The NMPs contained in the supernatant free of cytoskeletal contaminants were ethanol-precipitated and resuspended in the loading buffer used for high resolution 2-dimensional gel electrophoresis. The gels were run and analyzed as previously described (Wray et al., 1981).

RESULTS.

The aim of the proposed research was to assess the importance of nuclear organization for the regulation of cell behavior and the development of tumorigenesis. Two aspects of nuclear organization were studied during the first year of research: the loss or gain of specific nuclear matrix proteins during tumor transformation (2D gel analysis) and the role of the supramolecular organization of nuclear matrix proteins in the regulation of cell phenotype.

In order to work in conditions that recapitulate behaviors similar to the situation *in vivo*, we used a reconstituted basement membrane (rBM)-directed model of mammary gland morphogenesis and tumorigenesis and the HMT-3522 progression series. The HMT-3522 human mammary epithelial S1 cells were isolated from reduction mammoplasty and became immortalized in culture (Briand et al., 1987). Pre-malignant S2 cells, derived from S1 cells, were obtained by removing epidermal growth factor from the culture medium. Continuous passaging of S2 cells ultimately gave rise to tumor in nude mice. Epithelial T4 tumor cells were obtained from tumors given by S2 cells at passage 238. T4 tumor cells were further propagated as 2D monolayer (Briand et al., 1996). When embedded within a rBM, S1 cells show a phenotypically normal behavior. They arrest growth, organize an endogenous basement membrane (BM) and form polarized acinus-like structures with vectorial secretion of sialomucin into a central lumen (Petersen et al., 1992). Whereas, S2 cells (utilized in the range of passages preceding the acquisition of tumorigenic phenotype) form enormous cell assemblies in 3D culture, in which cells keep proliferating. T4 tumor cells cultured in 3D form tumor-like assemblies in which cells keep proliferating and invade the surrounding ECM. When the progression series is cultured in 2-dimensions (2D monolayer), none of the phenotypes described above are shown.

Nuclear matrix proteins as potential candidates for influencing tumor behavior.(unpublished data).

Using nuclear matrix preparations and 2D gel electrophoresis, a number of nuclear matrix-associated proteins (NMPs) have been found to be specific of tumor cell types and normal cell types.

However, none of the NMPs identified thus far have led to the development of new anticancer target (Nickerson 1998), and only one NMP is now in clinical trial to be used as a potential bladder tumor marker (Dr. Getzenberg, personal communication). The identification of potentially interesting NMPs is partly impaired by the time consuming search for meaningful changes observed when comparing 2D gels. The models used to look for interesting NMPs include the comparison of unrelated tumor cell types and the utilization of 2D cultures of cells that recapitulate cellular behaviors different from the situation *in vivo*. In these conditions, 2D gels show a large amount of different proteins that appear·or disappear when comparing normal and tumor cells. The chances to choose in the first attempt the right protein to study are weak.

We have tried to narrow down the possibilities to identify meaningful changes on 2D gel analysis of NMPs, by investigating the pattern of NMPs found in the HMT-3522 progression series. In collaboration with the laboratory of Dr. R. Getzenberg, we have studied the pattern of NMPs in S1 non-tumor cells, S2 pre-malignant cells, and T4 tumor cells, cultured both on 2D and in 3D. By only focusing on the NMPs that would disappear from S1 to S2 cells cultured in 3D and the NMPs that would appear from S2 cells to T4 cells cultured in 3D, we have identified three potential tumor suppressors and three potential tumor promoters. We believe that, since these NMPs were identified from *in vivo-* like behavior, the possibility that these proteins may be meaningful for the development of tumorigenesis is high. We also have recorded more changes in the pattern of NMPs by comparing intermediate steps in the progression series that may be useful to study specific aspects of tumor progression.

Nuclear organization is dependent on tissue structure.

The second aspect of the research project was to investigate the significance of NuMA protein distribution for the expression of cell behavior. My preliminary experiments had suggested that NuMA may respond to ECM signaling, because the rapid interruption of cell adhesion using trypsin or EDTA led to the collapse of NuMA to the center of the nucleus after nuclear matrix preparation, and because NuMA distribution was significantly different in non tumor vs tumor HMEC.

1) Changes in NuMA distribution accompany HMEC morphogenesis.
(See appendix: Lelièvre et al., 1998).

In order to assess if alteration in NuMA distribution accompanied important changes in cell behavior, we studied the nuclear pattern of NuMA during ECM-induced morphogenesis of S1 cells and compared this pattern to the distribution observed in cells cultured on 2D. When the cells are proliferating NuMA is diffusely distributed in the nucleus, however when the cells arrest their growth, NuMA redistribute into small nuclear foci. Interestingly, when we compare the distribution of NuMA in cell growth-arrested in 2D culture (after EGF removal) and cells growth arrested during the normal process of morphogenesis, we see subtle differences (figure 1). this indicates that the process of growth arrest may be slightly different in 2D and 3D cultures. Such hypothesis is confirmed by the fact that the distribution of cell cycle regulator Rb, another nuclear matrix protein, is different in 2D compared to 3D cultures. Finally, after completion of morphogenesis in S1 cells, NuMA is distributed in a few large nuclear foci (Figure 2). This distribution pattern was not observed in 2D cultures or in collagen I 3D cultures where the cells growth-arrest but do not undergo complete morphogenesis (figure 1). This indicates that the formation of large NuMA foci is a characteristic of ECM-induced morphogenesis. Interestingly large NuMA foci totally co-localized with large RNA splicing factor speckles, indicating that the reorganization of NuMA distribution

is somehow associated with the reorganization of the transcriptional machinery during morphogenesis. This latter result was confirmed by immunogold labeling for NuMA in electron microscopy, which showed NuMA in large interchromatin granules- structures known to contain a high concentration of RNA splicing factors (collaboration with the laboratory of Edmond Puvion, France).

In summary nuclear organization is remodeled during HMEC morphogenesis and the formation of tissue-like structure. The different stages of morphogenesis are associated with different distribution patterns of nuclear matrix proteins (Scheme 1).

2) Modulation of the capacity to undergo acini morphogenesis alters NuMA organization. (Unpublished results, manuscript in preparation).

To confirm that the nuclear distribution of NuMA was dependent upon HMEC acini morphogenesis, we looked at cells in which the morphogenic process was either impaired or re-established. S1 cells transfected with EGF receptor do not undergo proper morphogenesis (Wang et al., 1998). In this case NuMA does not distribute into large and distinct foci. Whereas, when tumor cells are forced to undergo morphogenesis by blocking the beta1- integrin signaling pathway (Weaver et al., 1997) or altering the MAP kinase signaling pathway (Wang et al., 1998), NuMA is found in large foci upon completion of morphogenesis (data not shown).

These results indicate that NuMA distribution pattern is a marker of HMEC differentiation.

3) The relationship between NuMA and ECM signaling. (Unpublished results).

Although the two precedent sections show that NuMA distribution is dependent on ECM signaling, it does not prove that there is a direct link between NuMA behavior and ECM signals. However, in the research proposal, I had presented a preliminary result showing that the alteration cell adhesion modifies NuMA distribution observed in nuclear matrix preparation. In order to dissect out the signaling pathways that may alter NuMA behavior, I studied the effect of directly altering the cytoskeleton, since the organization of the cytoskeleton is known to be regulated by cell adhesion. I treated S1 cells after they have undergone morphogenesis with either cytochalysin D, to disrupt actin microfilaments, colchicine, to disrupt microtubules, or specific antibodies against cytokeratins that induced the disruption of the intermediate filament network in cells permeabilized *in vivo*. Only alteration of the intermediate filament network induced a redistribution of NuMA, as shown by the 'migration' of the large foci to the center of the nucleus (Figure 3). This shows that the organization of intermediate filaments is important for the integrity of the nuclear organization of NuMA. However, this distribution pattern was slightly different from the pattern observed after disruption of cell adhesion. This indicates that additional changes are required in order to totally disrupt NuMA organization. This hypothesis is confirmed by the fact that, upon disruption of cell adhesion, the N-terminus part of NuMA protein is proteolized, as shown by the appearance of a lower molecular weight band on Western blots compared to the usual 220-240 kD bands (not shown). This proteolysis was not observed after alteration of the organization of the different cytoskeletal components. In addition, no alteration in NuMA phosphorylation could be detected in any of these conditions. Interestingly, the proteolysis of NuMA following disruption of cell adhesion in S1 cells that have undergone morphogenesis, was not observed for other proteins shown to redistribute during ECM-induced morphogenesis (e.g. splicing factors, Rb).

In summary, the results presented above confirm there may exist a privileged relationship between NuMA behavior and ECM signaling. We are now investigating the biochemical transduction pathways that may be associated with such a relationship.

Nuclear organization directs cell and tissue phenotype.
(See appendix, Lelièvre et al., 1998).

After showing that the distribution of specific nuclear matrix proteins changes in association with the expression of defined cell behaviors and the formation of tissue-like structure, the next important step was to assess if nuclear organization had a role to play in the determination of cellular and tissue phenotype. In order to answer this question, I disrupted specific nuclear structures after S1 cells had undergone morphogenesis in 3D. First, I altered chromatin structure by inducing histone hyperacetylation using trichostatin A. This treatment induced the cells to proliferate. It was also associated with the disruption of NuMA organization and the loss of basement membrane integrity (Figure 4). Second, in another set of experiments, I specifically altered NuMA organization by introducing anti-NuMA antibodies into living cells in the acini. Disruption of NuMA organization resulted in the diffusion of the protein throughout the nucleus. Most importantly, the targeted disruption of NuMA organization was accompanied by the alteration of chromatin structure- as shown by changes in the pattern of histone4 acetylation, and the induction of metalloproteases that were responsible for the degradation of the basement membrane (Figure 4).

These results are the first demonstration that the organization of non chromatin components of the nucleus can direct cellular and tissue phenotype. They also show there is a dynamic reciprocity between chromatin structure, the supramolecular organization of nuclear matrix proteins, and cell and tissue phenotype.

DISCUSSION.

The results presented above answer part of the aims of the proposed research. The aims 1 and 2 were to identify NMPs that may be implicated in tumorigenesis. We have six potential candidates and a few more candidate proteins that may be important for intermediate steps of tumor transformation. We are now trying to confirm these results, then we will move on to produce enough material in order to determine the sequence and localization of these candidate NMPs, as proposed in the second part of aims 1 and 2. This part of the work is taking time because of the number of comparisons that had to be done between the various steps of tumor transformation available in the progression series, all cultured both in 2D and in 3D. The use of the computerized processing of 2D gels established in Dr. Getzenberg's laboratory was here indispensable. Even though the comparison study was the most difficult ever attempted by Dr. Getzenberg, the results are encouraging and the collaboration continues.

The aim 3 of the proposal was to study the relationship between NuMA distribution and the cellular phenotype. The first part of the results is now in press in the Proceedings of National Academy of Sciences. The manuscript presents the first demonstration that nuclear organization depends on tissue structure (as illustrated by the study of acini morphogenesis) and that nuclear organization itself can direct cell and tissue phenotype. These data shed a new light on the understanding we have of the function of nuclear structure. Our results also present NuMA as an important protein for the differentiation of HMEC. Its function seems to be related to its different distribution patterns. We are now investigating the relationship between NuMA organization and gene expression in HMECs.

The part of the work proposed in aim 3 which more directly deals with the relation between NuMA distribution and ECM signaling is in progress. We have here reported that cytoskeleton organization is important but not sufficient to explain how cell membrane information may be

transmitted to NuMA, and that biochemical pathways may be essential for this signaling. This is not surprising since the regulation of biochemical pathways and the organization of the cytoskeleton have been shown to be interdependent (for a review see Lelièvre and Bissell, 1998). We are now investigating the participation of specific transduction pathway in the modulation of NuMA organization.

We did not used S1 cells transfected with stromelysin 1 gene as proposed in the research project. For unknown reasons this transfection was toxic for the cells after a while. We have however studied the relationship between NuMA and the ECM (basement membrane) in another way, since we could induce the activation of metalloproteases by disrupting NuMA organization. We are now investigating this surprising relationship by transfecting truncated forms of NuMA in our cells under an inducible promoter.

Another point in the proposal has been put aside for the moment. We wanted to identify if the relationship between NuMA and ECM signaling was due to direct signaling by cell-ECM adhesion complexes or by cell-cell adhesion complexes. Recent data obtained in the Bissell laboratory have demonstrated the existence of a coupling between different types of cell adhesion complexes, as well as between cell adhesion complexes and growth factor receptors when cells are grown in physiologically relevant conditions (3D cultures) (Weaver et al., 1997; Wang et al., 1998). It is therefore extremely difficult to find out the origin of the regulation pathways implicated in the regulation of nuclear organization and function. As a confirmation to the fact, an increasing number of drugs or components thought to be specific of one type of cell membrane receptor are now found to affect other types of cell membrane receptors. Thanks to electron microscopy studies, we have however some very preliminary data that may help decipher the cell membrane-mediated pathways associated with the regulation of NuMA.

The last part of specific aim 3, was to study the relationship between NuMA and the NMPs identified as potentially interesting for the regulation of HMEC behavior. This part of the project is waiting for the development of antibodies against the candidate NMPs.

During the first year of the research project, I have followed the objectives proposed in the statement of work. Next year will be devoted in pursuing the proposed objectives and developing targeted studies as a result of the data thus far obtained. More particularly we will develop molecular biology studies to better understand the role of NuMA in the regulation of gene expression and cell phenotype. This studies will also help decipher the transduction pathways involved in the reciprocal signaling between NuMA and the cell membrane.

CONCLUSION.

The results presented in this report suggest that the non chromatin structure of the nucleus may play an essential role in determining cell phenotype, as shown by the identification of potential tumor promoters and tumor suppressors among NMPs, and the demonstration that alteration of nuclear organization modifies cellular behavior. Notably, we have unraveled a reciprocal interaction between the chromatin structure, the supramolecular organization of NuMA, and the phenotype of HMECs.

We believe that understanding how nuclear structural components such as NuMA participate in signal transduction and regulation of gene expression will open new avenues for the design of novel anticancer therapy. NuMA may be one of the first candidates in the search for targeted therapy for breast cancer, which would for instance be based on induction of differentiation and/or promotion

of growth-arrest. Moreover our approach may lead to the development of pilot studies to look for other nuclear structural proteins behaving like NuMA.

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Figure 1. Effect of growth status on the distribution of NM proteins. Confocal fluorescence images (0.2μ optical sections) of NuMA (a-c) and Rb (d,e,g,h) in cells proliferating as 2D monolayers (a & d) and within 3D rBMs (g), and cells growth-arrested in monolayer (b & e) and within collagen-I (c) or a rBM (h). NuMA was diffusely distributed in the nucleus of proliferating HMECs grown as monolayers (a) and reorganized into random aggregates upon growth-arrest induced by EGF removal (b; the settings for image recording were the same as for image a; aggregates appear in white due to saturation of the signal). NuMA was distributed in random aggregates or in small foci in growth-arrested and BM-free cell colonies obtained after 10 days of culture within collagen-I (c). Rb was diffusely distributed in the nucleus of proliferating cells grown either in monolayer (d) or in 3D rBM (g), however, upon growth-arrest the protein redistributed into several foci in the monolayer propagated cells (e) but coalesced into a central, single nuclear focus in the rBM-induced acini (h; the dotted line indicates outer nuclear limit). Western blot analysis of Rb in proliferating and growth-arrested cells grown as monolayers (f) or within a 3D rBM (i) shows that the hyperphosphorylated isoform was only present in proliferating cells. Scale bar represents 10μ. Arrows indicate nuclei.

Figure 2. Nuclear matrix protein redistribution in HMECs following 3D rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2μ optical sections) of lamin B, NuMA and splicing factor SRm160 in cells grown as monolayers (2D, a-c) and within rBMs (3D, d-i). NuMA was diffusely distributed in the nuclei of cells grown as monolayers (b), but reorganized into large nuclear foci in cells induced to undergo morphogenesis (acini formation) in response to a rBM (e). SRm160 was distributed as multiple nuclear speckles in cells cultured as monolayer (c), whereas it was concentrated into fewer and larger speckles in the acini (f). Lamin B in contrast, consistently localized to the nuclear periphery and within intra nuclear patches (a & d). The distribution of lamin B (g), NuMA (h) and SRm160 (i) after *in situ* NM preparation of cells cultured in 3D rBM was similar to that observed in intact cells (d-f). Scale bar 10μ. Arrows indicate nuclei found within the plane of the section.

Figure 3. Alteration of NuMA distribution in nuclear matrix preparation following disruption of cell adhesion and cytoskeletal organization. NuMA is organized in a few large foci in the nucleus of S1 cells that have undergone morphogenesis (acini formation) (control, A). When cell adhesion was disrupted after short *in vivo* treatment of acini with trypsin or EDTA, NuMA coalesced to the center of the nucleus (B). This phenomenon is reversible after a few hours. When acini were treated *in vivo* with acrylamide (C) or anti-cytokeratin antibodies (following digitonin permeabilization) (D), that disrupted intermediate filaments organization, NuMA foci also coalesced to the center of the nucleus, although less completely than following direct disruption of cell adhesion. The schemes under each picture show NuMA organization in the nucleus.

Figure 4. Cross-modulation between chromatin structure, NM organization and the acinar phenotype. Confocal fluorescence images (0.2 μ optical sections) of NuMA (a,e,i), collagen IV (b,f,j), β-catenin (c,g,k) and acetylated histone H4 (d,h,l) in control, trichostatin A (TSA)-treated and NuMA monoclonal antibody (mAb)-incubated acini (day 10 of 3D rBM culture). (a-d) Nuclear organization and acinar phenotype in controls: acini exhibit NuMA foci (a), an organized

endogenous collagen IV-rich BM (b), cell-cell localized β-catenin (c) and dispersed acetylated H4 histone (d). (e-h) Effects of TSA on nuclear architecture and acinar phenotype: following 24 hours of TSA treatment (40nM), more than 55 percent of the cells entered the cell cycle, as indicated by an increase in Ki-67 labeling index (m) and the appearance of mitotic cells (e; arrow). NuMA was uniformly distributed in the nuclei (e), collagen IV disappeared (f), β-catenin was released from the cell-cell interface (g), and the pattern of histone H4 acetylation was altered (h). (i-l) Effects of mAb-induced NuMA foci disruption on nuclear organization and acinar phenotype: introduction of a NuMA mAb into the nuclei of the acini, using reversible digitonin permeabilization, led to the disruption of NuMA foci (i), degradation of the collagen IV-rich BM (j; arrows), and the nuclear marginalization of acetylated H4 histone (l). There was no consistent alterations observed for β-catenin other than increased basal labeling (k). These effects were not observed using mock IgG's or mAbs to lamins A/C or B. (n) BM degradation following mAb-induced NuMA disruption in acini: analysis of the percentage of acini with intact collagen IV-rich BMs in relation to (a) control/digitonin-permeabilized (DP) acini, (b) mock-IgG mAb-treated/DP acini, (c) NuMA mAb-treated/non-permeabilized acini, (d) NuMA mAb-treated/DP acini, (e) NuMA mAb-treated/DP acini + the metalloproteinase inhibitor GM6001, (f) NuMA mAb-treated/DP acini + the inactive metalloproteinase inhibitor GM1210, (g) NuMA mAb-treated/DP acini + the uPA inhibitor, aprotinin, and (h) Lamin B mAb-treated/DP acini. More than 35 percent of acini degraded their endogenous BMs in response to disruption of NuMA (d). The BM loss could be rescued by treatment with the metalloproteinase inhibitor GM6001 (e), but not its inactive analogue (f) or a uPA protease inhibitor (g). Scale bar 10μ.
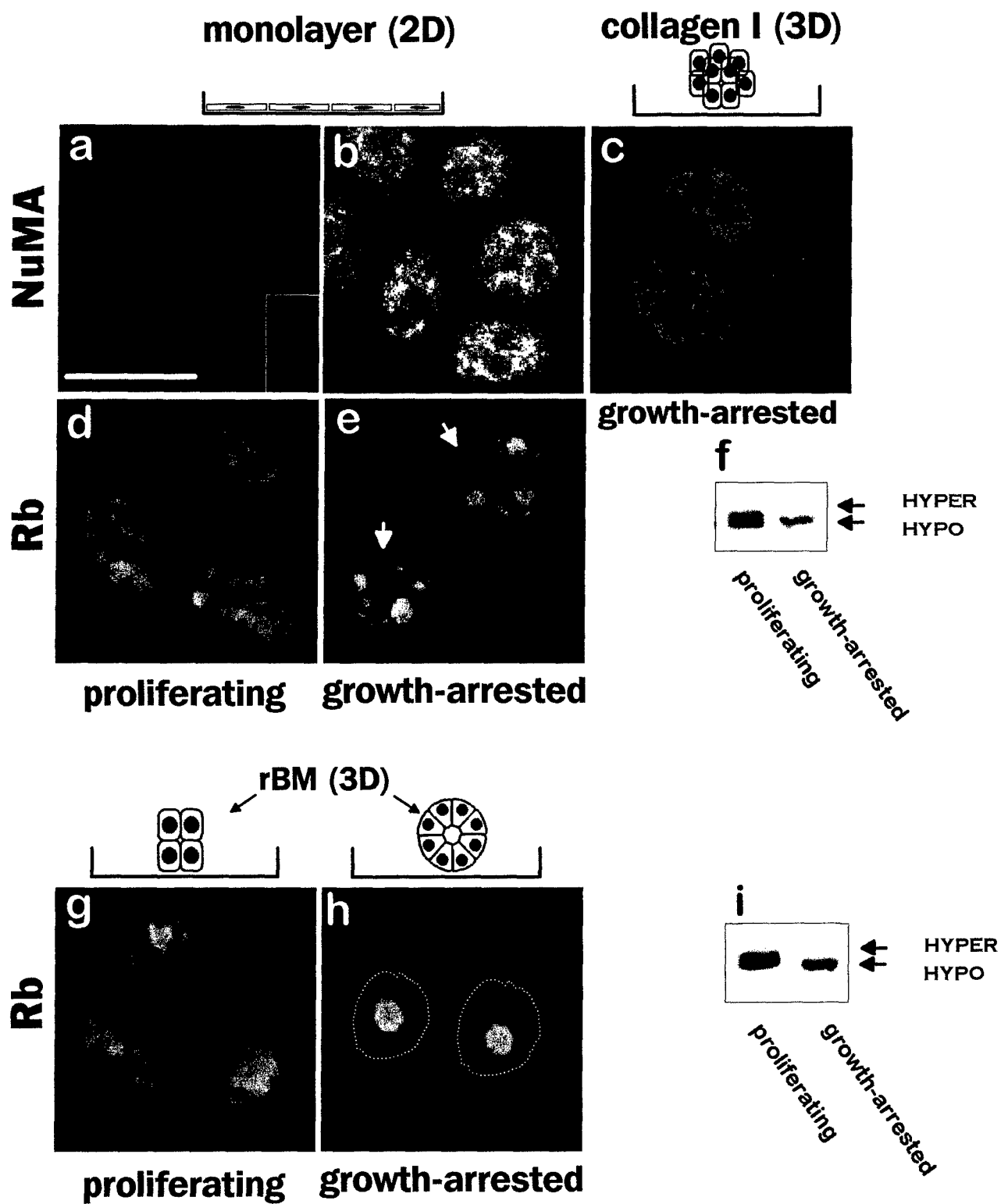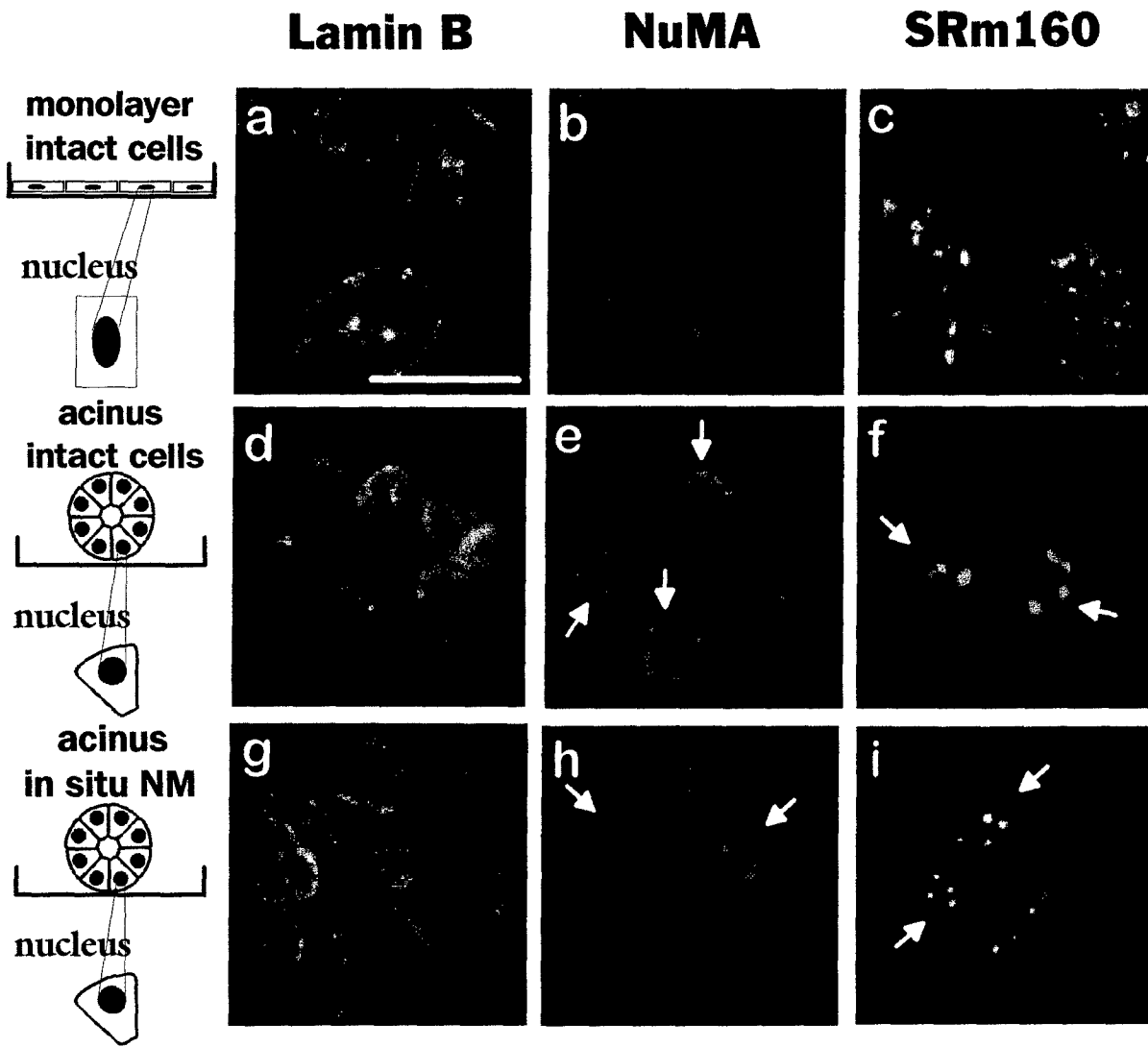
monolayer (2D)   collagen I (3D)

NuMA

a   b   c

growth-arrested

Rb

d   e   f

HYPER
HYPO

proliferating   growth-arrested

proliferating
growth-arrested

rBM (3D)

Rb

g   h   i

HYPER
HYPO

proliferating   growth-arrested

proliferating
growth-arrested

Figure 1

Figure 2

Figure 3

Figure 4

Dynamics of the distribution of NM proteins in 3D rBM
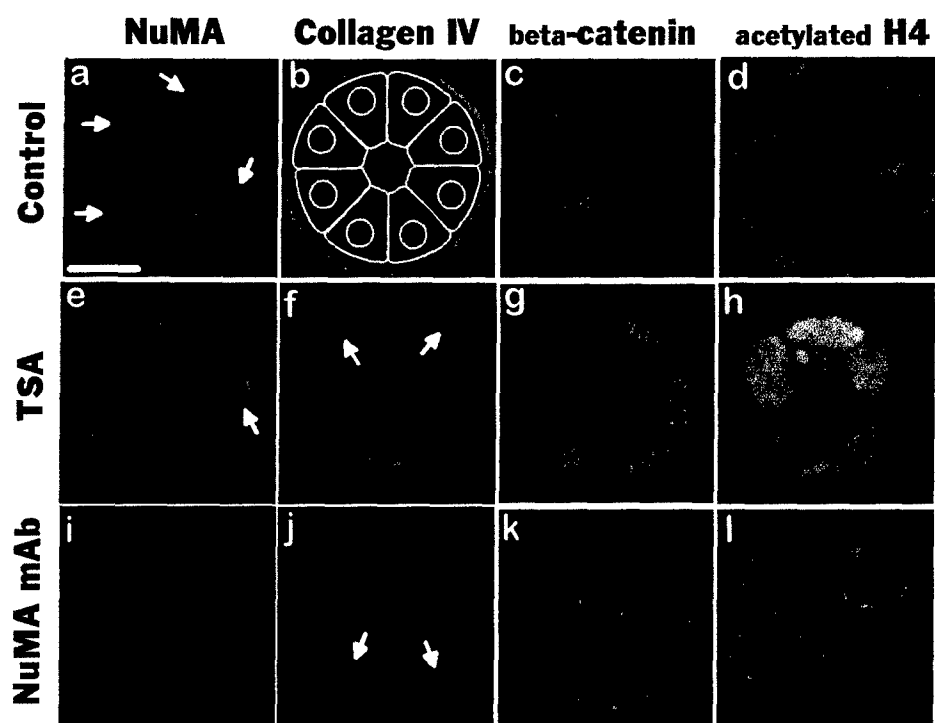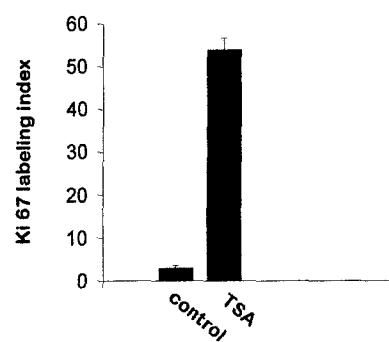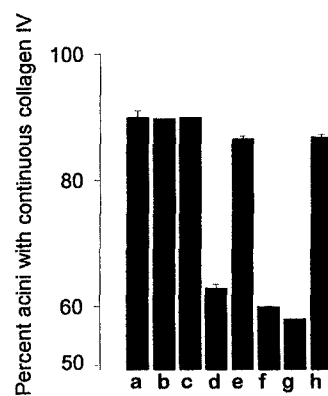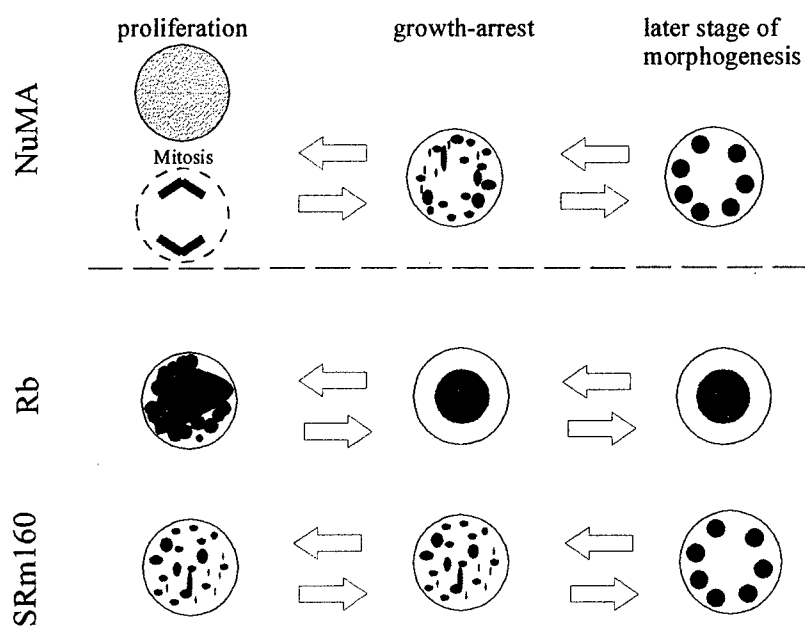

(Only the cell nucleus is represented, NuMA in red; Rb and SRm160 in green)

Scheme 1

CLASSIFICATION - Biological Sciences: Cell Biology

Tissue Phenotype Is Dependent on Reciprocal Interactions Between the Extracellular Matrix and the Structural Organization of the Nucleus.

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Abbreviations: NM, nuclear matrix; rBM, reconstituted basement membrane; HMEC, human mammary epithelial cells; 2D and 3D, two and three dimensional; Rb, retinoblastoma protein; ECM, extracellular matrix; EGF, epidermal growth factor; mAb, monoclonal antibody.

ABSTRACT

What determines the nuclear organization within a cell and whether this organization itself can impose cellular function within a tissue remains unknown. To explore the relationship between nuclear organization and tissue architecture and function, we used a model of human mammary epithelial cell acinar morphogenesis. When cultured within a reconstituted basement membrane (rBM), HMT-3522 cells form polarized and growth-arrested tissue-like acini with a central lumen, and deposit an endogenous BM. We show that rBM-induced morphogenesis is accompanied by re-localization of the nuclear matrix proteins NuMA, splicing factor SRm160, and cell cycle regulator Rb. These proteins had distinct distribution patterns specific for proliferation, growth-arrest and acini formation, whereas the distribution of the nuclear lamina protein, lamin B, remained unchanged. NuMA re-localized to foci which coalesced into larger assemblies as morphogenesis progressed. Perturbation of histone acetylation in the acini by trichostatin A treatment altered chromatin structure, disrupted NuMA foci and induced cell proliferation. Moreover, treatment of transiently permeabilized acini with a NuMA antibody led to the disruption of NuMA foci, alteration of histone acetylation, activation of metalloproteases, and breakdown of the endogenous BM. These results are the first experimental demonstration of a dynamic interaction between the extracellular matrix, nuclear organization and tissue phenotype. They further show that rather than passively reflecting changes in gene expression, nuclear organization itself can modulate the cellular and tissue phenotype.

INTRODUCTION

The cell nucleus is organized by a non-chromatin internal structure referred to as the nuclear matrix (NM) (1-3). Identified NM components include coiled-coil proteins (4), cell cycle regulators (5), tissue-specific transcription factors (6-7), and RNA splicing factors (for review see 2). Although splicing factors have been shown to redistribute during cellular differentiation (8-9), and following the induction of gene expression (10), spatial distribution of nuclear components are thought to be the consequence of changes in gene expression (8, 10-11). However, whether NM composition and structure may themselves affect gene expression and cellular function has not been examined.

To systematically study the effect of cell growth and tissue differentiation on nuclear organization, we used a reconstituted basement membrane (rBM)-directed model of mammary gland morphogenesis (12). The HMT-3522 human mammary epithelial cells (HMECs) were isolated from reduction mammoplasty and became immortalized in culture (13). When embedded within a rBM, these cells arrest growth, organize an endogenous BM and form polarized acinus-like structures with vectorial secretion of sialomucin into a central lumen (12). Using this model, we have compared the nuclear organization of HMECs cultured on a plastic surface (2D monolayer) *vs* a 3-dimensional (3D) rBM. Nuclear organization was assessed by examining the distribution of the coiled-coil NM proteins lamin B (14) and NuMA (15), the cell cycle regulator Rb (p110Rb; 5), and the splicing factor SRm160 (formerly known as B1C8; 16). These proteins had distinct spatial distribution patterns specific for proliferation, growth-arrest and acini formation. Moreover, disruption of nuclear organization in acini by either perturbing histone acetylation, or directly modifying the distribution of NM proteins, altered the acinar phenotype.

We previously hypothesized (17), and thereafter provided evidence that the extracellular matrix (ECM) directs morphogenesis and gene expression in mammary epithelial cells (12;18-19). Here we show that a reciprocal relationship exists between the ECM and nuclear organization. These findings underscore a role for nuclear organization in regulation of gene expression and provide a possible framework for how cell-ECM interactions determine cell and tissue phenotype.

MATERIAL AND METHODS

Cell Culture

HMT-3522 HMECs (S1 passage 50; 13) were propagated in 2D cultures in chemically defined medium (12) and growth-arrest was induced by removing epidermal growth factor (EGF) for 48 hours. Cultures were prepared by embedding single cells (8.5×10^5 cells/ml matrix) in rBM (Matrigel™, Collaborative Research) or collagen-I matrix (Cellagen™ AC-5, ICN Biochemicals Incorporated) in four-well chamber slides (Nalge Nunc International). These cultures were grown for 5-10 days. Growth-arrest and morphogenesis were routinely observed by days 7-9.

Antibodies and inhibitors

For Western blots and/or immunostaining, we used monoclonal antibodies (mAbs) against type IV collagen (clone CIV, Dako), β-catenin (clone 14, Transduction Laboratories), SRm160 splicing factor (clone B1C8, 16), lamin B (clone 101-B7, Matritech Inc.), NuMA (clone 204-41, Matritech Inc., and clone B1C11, a gift from Dr. S. Penman), and polyclonal antibodies (Abs) against Ki-67 (Novocastra Laboratory), acetylated histone H4 (Upstate Biotechnology Incorporated), and p110Rb (Santa Cruz Biotechnology). For bioperturbation assays, we used mAbs against lamins A/C (clone 636, Novocastra Laboratory) and NuMA (clone 22,

Transduction Laboratory), in addition to B1C11 and 101-B7. Trichostatin A (Wako Chemicals) was used as an inhibitor of histone deacetylase (40 nM).

Indirect immunofluorescence

Cells were permeabilized *in situ* (0.5% triton-X 100 in 100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 5 mM MgCl2, containing 1 mM PefablocTM, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor type II, and 250 μM NaF), fixed in 2% paraformaldehyde, and immunostained as previously described (18). Human mammary tissue was snap-frozen in n-hexane and embedded in Tissue-Tek OCT (Miles laboratories); 5 μ sections were fixed in methanol and immunostained in accordance with human protocol (KF) 01-216/93 in the laboratory of Dr. O. Petersen.

Image acquisition, processing, and data analysis

Samples were analyzed using a Bio-Rad MRC 1024 laser scanning confocal microscope attached to a Nikon Diaphot 200 microscope. Fluorescence specificity was ensured by sequential fluorophore excitation. NuMA foci were analyzed using Image Space-3D analysis program (Molecular Probes) and normalized to 3D rBM cluster cell number by highlighting and counting each nucleus using Image Space-measure 2D. The voxel threshold was set at 0.2 μ.

Immunoblot analysis

Total cell extracts (2% SDS in phosphate buffered saline pH 7.4, containing 1 mM Pefabloc™, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor type II, and 250 μM NaF) were prepared *in situ* for 2D cultures, or from acini isolated from 3D cultures by dispase treatment (5,000 U/ml caseinolytic activity, Collaborative Research). Equal amounts of protein were separated and immunoblotted as previously described (22).

In situ NM preparation.

In situ NM preparation was as previously described (20), except that 0.05 % triton-X 100 and micrococcal nuclease (5 U/ml; Sigma) were used.

Antibody-mediated perturbation of nuclear organization.

rBM induced-acini (day 10) were permeabilized for less than two minutes *in situ* (0.01% digitonin in 25 mM Hepes pH 7.2, 78 mM KHOAc, 3 mM MgHOAc, 1 mM EGTA, 300 mM sucrose, and 1% RIA grade bovine serum albumin), rinsed twice in digitonin-free buffer, and incubated in medium containing dialyzed specific or mock Abs (15 µg/ml) for 48 hours, after which the cells were incubated with fresh medium for an additional 48 hours. Antibody concentrations and incubation times were determined empirically. Trypan blue dye exclusion tests and apoptosis studies verified the absence of digitonin toxicity.

RESULTS

Internal nuclear organization is remodeled when HMECs are cultured within a basement membrane.

HMT-3522 HMECs, like primary HMECs, undergo morphogenesis to form tissue-like acini when cultured in a 3D rBM (12;18). Neither cell type undergoes acinar differentiation when cultured as 2D monolayers. In proliferating 2D cultures, NuMA was diffusely distributed in the nucleus (Fig. 1b) except when localized to the spindle poles in mitotic cells (15), and splicing factor SRm160 was distributed into numerous speckles of heterogenous sizes (Fig. 1c; 16). In rBM-induced acini, NuMA was redistributed into an average of eight nuclear foci (ranging from 1 to 1.6 μ in diameter), surrounded by diffusely localized NuMA protein (Fig. 1e), and SRm160 was distributed into an average of seven large speckles (Fig. 1f). In contrast, lamin B maintained a peripheral ring-like distribution around the nucleus, with some internal localization, regardless of culture conditions (Fig. 1a and d). The distribution pattern of these proteins was conserved in NM preparations *in situ*, where chromatin was removed before immunolocalization (staining is shown for 3D rBM cultures only (Fig. 1g-i).

We next examined NuMA and SRm160 distribution at different stages of 3D rBM-induced morphogenesis. After embedment in rBM, cells proliferated to form small clusters by days 3-5, but lacked β-catenin at cell-cell junctions, and collagen IV staining was discontinuous (Fig. 2A. a-c). Following growth-arrest (days 6-10), cells assembled a continuous endogenous BM, and formed polarized acinus-like structures with organized adherens junctions (Fig. 2A. d-f). NuMA was uniformly distributed in the nuclei of proliferating cells (Fig. 2B. a), but became concentrated into distinct foci of differing sizes following growth-arrest (day 7; Fig. 2B. b), and into larger and fewer foci upon completion of morphogenesis (day 10, Fig. 2B. c). NuMA and the splicing factor SRm160 were not co-localized in proliferating cells (Fig. 2B. a' and a''), but NuMA foci and SRm160 speckles were closer together following growth-arrest (Fig. 2B. b' and

b''), and were completely co-localized in large assemblies after the completion of morphogenesis (Fig. 2B. c' and c''). These spatial changes in NuMA arrangement occurred without significant modifications in the level of NuMA expression or molecular weight, as determined by western blot analysis (Fig. 2B. e). These experiments demonstrate that specific NM proteins undergo spatial rearrangement during rBM-induced acinar morphogenesis. Since the existence of NuMA in differentiated tissue has been questioned (21), we studied NuMA in the normal resting human mammary gland. Intense staining was observed in the epithelial cells of acini and ducts where NuMA was distributed in foci of different sizes, resembling the acinar stages recapitulated in 3D rBM cultures (Fig. 2B. d).

Growth-arrest is associated with changes in NuMA and Rb distribution.

ECM-directed growth-arrest is an early and critical step in mammary epithelial cell morphogenesis (12). To distinguish between the effect of ECM-directed growth-arrest and changes due to tissue structure and polarity, the localization of NuMA and SRm160 was compared between growth-arrested and proliferating cells cultured in monolayers. Less than five percent of the cells remained in the cell cycle following growth-arrest induced by EGF removal, as indicated by the absence of detectable Ki-67 immunostaining (not shown). NuMA was uniformly distributed in the nuclei of proliferating cells, but coalesced into denser areas upon growth-arrest (Fig 3a-b). The irregular geometric quality of these dense areas was distinct from the circular foci pattern observed in growth-arrested 3D rBM-grown cells. In contrast, no significant change in the multi-speckled distribution of SRm160 was detected under these conditions (not shown). The relationship between nuclear organization and growth status was further investigated by examining the distribution of the cell cycle regulator Rb. Rb redistributed from a diffuse nuclear pattern in proliferating HMECs, into a few large foci in growth-arrested cells (Fig. 3d-e). Strikingly, the distribution of Rb in the growth-arrested 2D cultures was distinct

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from that observed in the growth-arrested 3D cultures (compare Fig. 3e to 3h), which may reflect differences in the state of growth arrest between 2D monolayer and 3D rBM cultures. The mono-focal pattern of Rb observed in 3D culture coincided with growth-arrest. Western blot analysis showed that hypophosphorylated Rb was associated with the NM in 3D cultures (not shown), as was previously reported for 2D cultures (5). Moreover, the diffuse distribution observed in proliferating cells was associated with the hyperphosphorylated form of the protein (Fig. 3f and 3i).

Since growth-arrest in 3D rBM precedes the final stages of acinar morphogenesis (13), we examined the relationship between the large NuMA foci and the formation of a polarized endogenous BM. HMECs cultured in a 3D collagen-I matrix form growth-arrested organized colonies, but do not assemble a polarized, endogenous BM (22). Therefore, we compared NuMA distribution in cells grown in rBM to those grown in type I collagen. After 12 days in collagen I, NuMA was distributed as small foci or irregular dense aggregates (Fig 3c), similar to the pattern observed in growth-arrested cells in 2D cultures. Thus, NuMA redistribution into dense areas and small foci is induced by growth-arrest, but the coalescence of the foci into larger and distinct structures requires the presence of a BM.

Cross-modulation between NuMA distribution, chromatin structure, and the acinar phenotype.

The degree of histone acetylation has been shown to regulate chromatin structure and gene expression (23,19). Histone acetylation was altered in the acini using the histone deacetylase inhibitor trichostatin A. After two hours of treatment, NuMA foci began to disperse, and several cells entered the cell cycle, as measured by an increase in the Ki-67 labeling index. After 24 hours of treatment, NuMA was diffusely distributed in all nuclei (Fig. 4e compared to 4a), and the acinar phenotype was altered as shown by loss of the endogenous BM (Fig. 4f

compared to 4b), redistribution of β-catenin (Fig. 4g compared to 4c), and the presence of mitotic cells, shown by mitotic spindle pole staining of NuMA (Fig. 4e, arrowhead). In contrast, trichostatin A did not alter the cell phenotype, or the distribution of NuMA (not shown).

Since NuMA is essential for nuclear assembly following mitosis and participates in the loss of nuclear integrity during apoptosis (24-25), we asked whether disruption of NuMA foci in the acini could globally influence nuclear organization and affect the acinar phenotype. Rapid and reversible digitonin permeabilization was used to load cells with either anti-NuMA mAbs, or with an IgG1 mock mAb. The NuMA mAb B1C11, but not an N-terminal-specific mAb (clone 22) (not shown), disrupted NuMA organization, causing the protein to become diffusely redistributed within the nucleus, as revealed by the secondary Ab (Fig. 4i). Chromatin structure was altered, as shown by the rearrangement of acetylated histone H4 distribution (compare Fig. 4L with 4d). More dramatically, disruption of NuMA organization altered the acinar phenotype, indicated by loss of the endogenously deposited BM (Fig. 4j). Since the loss could be prevented by treatment with GM6001, a potent metalloprotease inhibitor (Fig. 4n; 26), we conclude that NuMA disruption led to induction and/or activation of a metalloprotease. Similar treatment of the acini with mAbs against lamins A/C or lamin B did not induce any change in histone H4 acetylation, BM integrity or lamin distribution, even though these Abs reached their nuclear targets, as shown by secondary Ab staining (Fig 4n and not shown).

DISCUSSION

By modifying the cellular microenvironment, we have demonstrated that nuclear organization rearranges dramatically in HMECs following growth-arrest and tissue-like acinar morphogenesis (scheme 1). The use of the 3D rBM culture assay has enabled us also to show that alterations of nuclear organization can modify the cellular and tissue phenotype.

Thus far documented changes in nuclear organization have been broadly descriptive. By systematically analyzing the distribution of three NM proteins in 2 and 3D cultures, we have determined that precise nuclear rearrangements occur with growth-arrest and following rBM-induced morphogenesis. In 3D rBM cultures, both NuMA and Rb were diffusely distributed in the nucleus of proliferating cells. Following growth-arrest, NuMA was re-localized into discrete foci, while Rb redistributed into a central nuclear mass. These patterns of distribution were different from those observed in growth-arrested cells in monolayer 2D cultures, suggesting that there may be different states of growth-arrest in 2D and 3D rBM cultures (27). Since NuMA distribution in 3D collagen I cultures was comparable to that observed in growth-arrested 2D cultures, our results suggest that 3D organization of cells per se cannot explain the differences seen between monolayer and 3D-rBM cultures. The finding implies that BM signaling is necessary for the ultimate nuclear organization within the acini. Indeed, the presence of large and distinct NuMA foci was observed only in mature 3D rBM cultures, and was found also in adult resting mammary gland *in vivo,* where the acini were surrounded by a continuous endogenous BM. The mammary gland undergoes developmental cycles of growth and differentiation even in adults; this may account for the heterogeneity of foci size observed *in vivo,* and may further explain the absence of the very large NuMA foci in subpopulations of differentiated 3D rBM-induced acini (Fig. 2B. c). Whether the pattern of NuMA distribution indeed corresponds to different levels of differentiation *in vivo* requires further analysis.

The antibody-directed disruption of NuMA foci in the acini induced changes in the distribution pattern of acetylated histone H4, the activation of metalloprotease(s) and the loss of BM integrity. These results, as well as our observation that NuMA progressively coalesces and eventually co-localizes with enlarged splicing factor speckles during acini differentiation, suggests that some nuclear proteins may contain the molecular information necessary for the development and/or maintenance of the acinar phenotype. Interestingly, trichostatin-induced alteration of histone acetylation in acini also led to the disruption of NuMA foci, and was associated with the loss of BM, and the induction of cell proliferation. Although we do not know the molecular mechanisms responsible for phenotypic alterations induced by nuclear reorganization, our experiments demonstrate the existence of reciprocal interactions also between nuclear organization, chromatin structure and the acinar phenotype. The BM has been shown previously to be necessary for the formation and maintenance of the functional acinus (12;28-29). We report here that BM-induced acinar formation is associated with the distinct spatial organization of a repertoire of NM proteins, and that conversely, perturbation of nuclear organization alters the BM, and influences the acinar phenotype. These results illustrate the dynamic reciprocity between the ECM and the structural organization of the nucleus, and underscore the importance of ECM-NM communication (17) in phenotypic plasticity.

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FIGURE LEGENDS.

Figure 1.   NM protein redistribution in HMECs following 3D rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2μ optical sections) of lamin B, NuMA and splicing factor SRm160 'in cells grown as monolayers (2D, a-c) and within rBMs (3D, d-i). NuMA was diffusely distributed in the nuclei of cells grown as monolayers (b), but reorganized into large nuclear foci in cells induced to undergo morphogenesis (acini formation) in response to a rBM (e). SRm160 was distributed as multiple nuclear speckles in cells cultured as monolayer (c), whereas it was concentrated into fewer and larger speckles in the acini (f). Lamin B in contrast, consistently localized to the nuclear periphery and within intra nuclear patches (a & d). The distribution of lamin B (g), NuMA (h) and SRm160 (i) after *in situ* NM preparation of cells cultured in 3D rBM was similar to that observed in intact cells (d-f). Scale bar 10μ. Arrows indicate nuclei found within the plane of the section.

Figure 2A.   Distribution of structural proteins during rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2μ optical sections) of collagen IV, β-catenin and Ki-67 in HMECs embedded within a rBM for 3-4 days (proliferating cells, a-c), and for 7-10 days (growth-arrested acini, d-f). Coincident with growth-arrest and acinar morphogenesis, HMECs deposited an organized endogenous collagen IV-rich BM (compare a with d), while β-catenin re-localized from the cytosol and basal plasma membrane to sites of cell-cell adhesion (compare b with e). Acinar morphogenesis was associated with cell-cycle exit, as indicated by the loss of Ki-67 staining (compare c with f). Scale bar 10μ.

Figure 2B.   Spatial analysis of NuMA and Splicing factor SRm160 redistribution during rBM- induced acinar morphogenesis. Confocal fluorescence images (0.2μ optical sections) of NuMA (Texas red, a-c), and double-labeled NuMA (Texas red), and SRm160 (FITC green)(a',a"-c',c") in HMT-3522 cells proliferating (a,a'.a"), and undergoing morphogenesis

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(b,b',b"& c,c',c") in response to a rBM. In proliferating cells NuMA was diffusely distributed (a) and did not co-localize with SRm160 (a' & a"). Following growth-arrest NuMA coalesced into foci of increasing size (0.2-2 μ, f) in association with the establishment of mature tissue-like structures (acini) (b-9 nuclei shown, & c). Only the larger NuMA foci observed in late morphogenesis fully co-localized with SRm160 (b', b"-c',c"). (d) In the ductal and acinar HMECs of the mammary gland, *in vivo,* NuMA was localized in foci with a size distribution comparable to that observed in most of the HMEC nuclei of differentiating rBM cultures (b). (e) Western blot analysis of NuMA (top) and Lamin B (bottom) showed no difference in protein expression or size between proliferating and growth-arrested HMECs grown within rBMs. Scale bar 10μ. Arrows indicate nuclei.

Figure 3.   Effect of growth status on the distribution of NM proteins. Confocal fluorescence images (0.2μ optical sections) of NuMA (Texas red, a-c) and Rb (FITC green, d,e,g,h) in cells proliferating as 2D monolayers (a & d) and within 3D rBMs (g), and cells growth-arrested in monolayer (b & e) and within collagen-I (c) or a rBM (h). NuMA was diffusely distributed in the nucleus of proliferating HMECs grown as monolayers (a) and reorganized into random aggregates upon growth-arrest induced by EGF removal (b; the settings for image recording were the same as for image a; aggregates appear in white due to saturation of the signal). NuMA was distributed in random aggregates or in small foci in growth-arrested and BM-free cell colonies obtained after 10 days of culture within collagen-I (c). Rb was diffusely distributed in the nucleus of proliferating cells grown either in monolayer (d) or in 3D rBM (g), however, upon growth-arrest the protein redistributed into several foci in the monolayer propagated cells (e) but coalesced into a central, single nuclear focus in the rBM-induced acini (h; the dotted line indicates outer nuclear limit). Western blot analysis of Rb in proliferating and growth-arrested

cells grown as monolayers (f) or within a 3D rBM (i) shows that the hyperphosphorylated isoform was only present in proliferating cells. Scale bar represents 10μ. Arrows indicate nuclei.

Figure 4. Cross-modulation between chromatin structure, NM organization and the acinar phenotype. Confocal fluorescence images (0.2 μ optical sections) of NuMA (a,e,i), collagen IV (b,f,j), β-catenin (c,g,k) and acetylated histone H4 (d,h,l) in control, trichostatin A (TSA)-treated and NuMA monoclonal antibody (mAb)-incubated acini (day 10 of 3D rBM culture). (a-d) Nuclear organization and acinar phenotype in controls: acini exhibit NuMA foci (a), an organized endogenous collagen IV-rich BM (b), cell-cell localized β-catenin (c) and dispersed acetylated H4 histone (d). (e-h) Effects of TSA on nuclear architecture and acinar phenotype: following 24 hours of TSA treatment (40nM), more than 55 percent of the cells entered the cell cycle, as indicated by an increase in Ki-67 labeling index (m) and the appearance of mitotic cells (e; arrow). NuMA was uniformly distributed in the nuclei (e), collagen IV disappeared (f), β-catenin was released from the cell-cell interface (g), and the pattern of histone H4 acetylation was altered (h). (i-l) Effects of mAb-induced NuMA foci disruption on nuclear organization and acinar phenotype: introduction of a NuMA mAb into the nuclei of the acini, using reversible digitonin permeabilization, led to the disruption of NuMA foci (i), degradation of the collagen IV-rich BM (j; arrows), and the nuclear marginalization of acetylated H4 histone (l). There was no consistent alterations observed for β-catenin other than increased basal labeling (k). These effects were not observed using mock IgG's or mAbs to lamins A/C or B. (n) BM degradation following mAb-induced NuMA disruption in acini: analysis of the percentage of acini with intact collagen IV-rich BMs in relation to (a) control/digitonin-permeabilized (DP) acini, (b) mock-IgG mAb-treated/DP acini, (c) NuMA mAb-treated/non-permeabilized acini, (d) NuMA mAb-treated/DP acini, (e) NuMA mAb-treated/DP acini + the metalloproteinase inhibitor GM6001, (f) NuMA mAb-treated/DP acini + the inactive metalloproteinase inhibitor GM1210,

(g) NuMA mAb-treated/DP acini + the uPA inhibitor, aprotinin, and (h) Lamin B mAb-treated/DP acini. More than 35 percent of acini degraded their endogenous BMs in response to disruption of NuMA (d). The BM loss could be rescued by treatment with the metalloproteinase inhibitor GM6001 (e), but not its inactive analogue (f) or a uPA protease inhibitor (g). Scale bar 10μ.
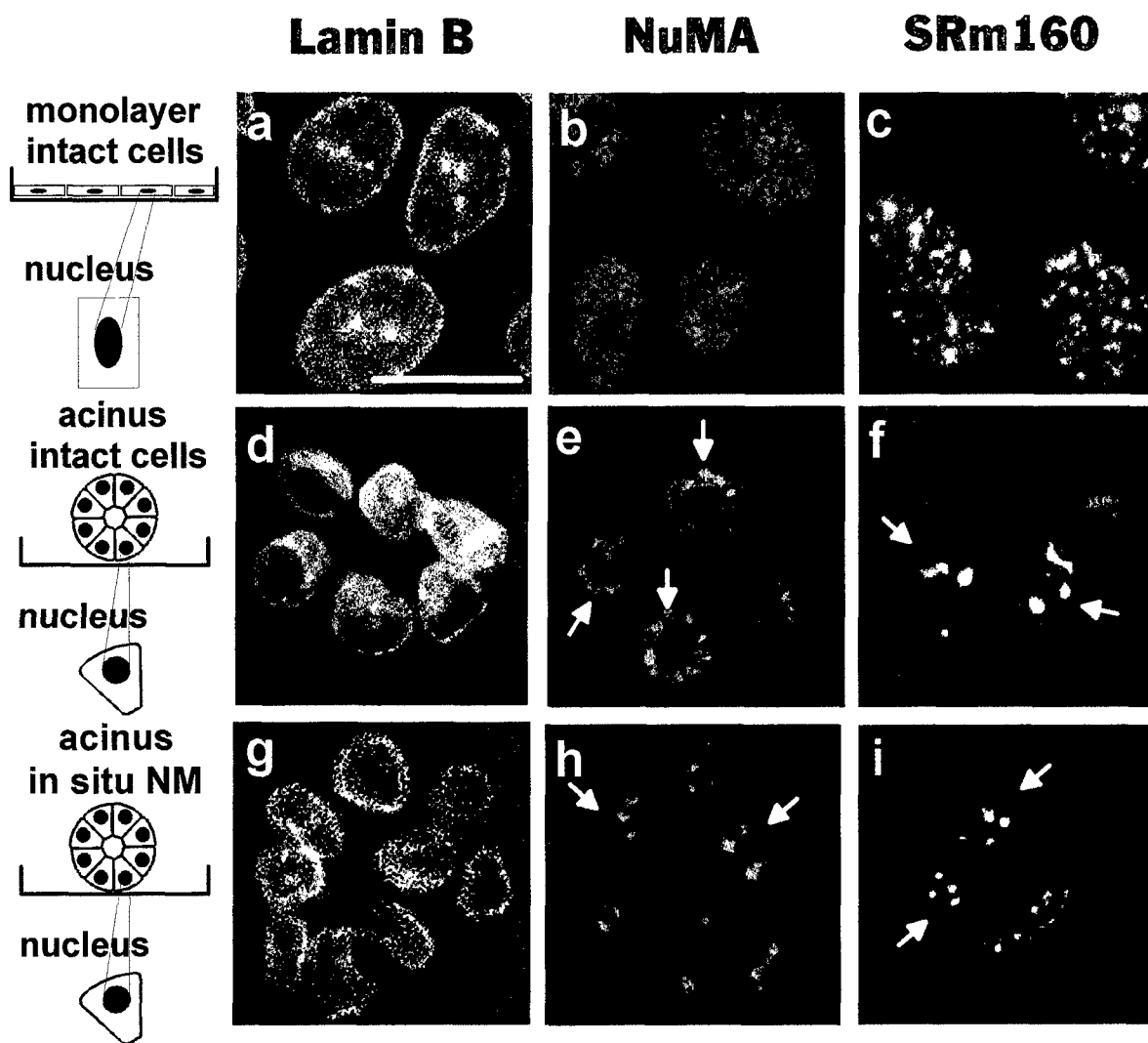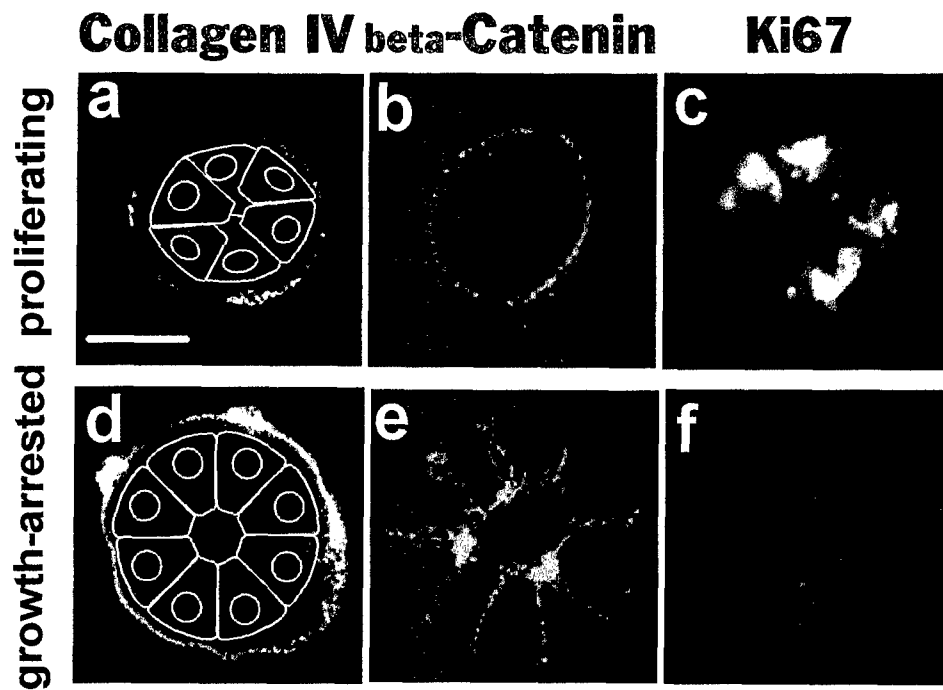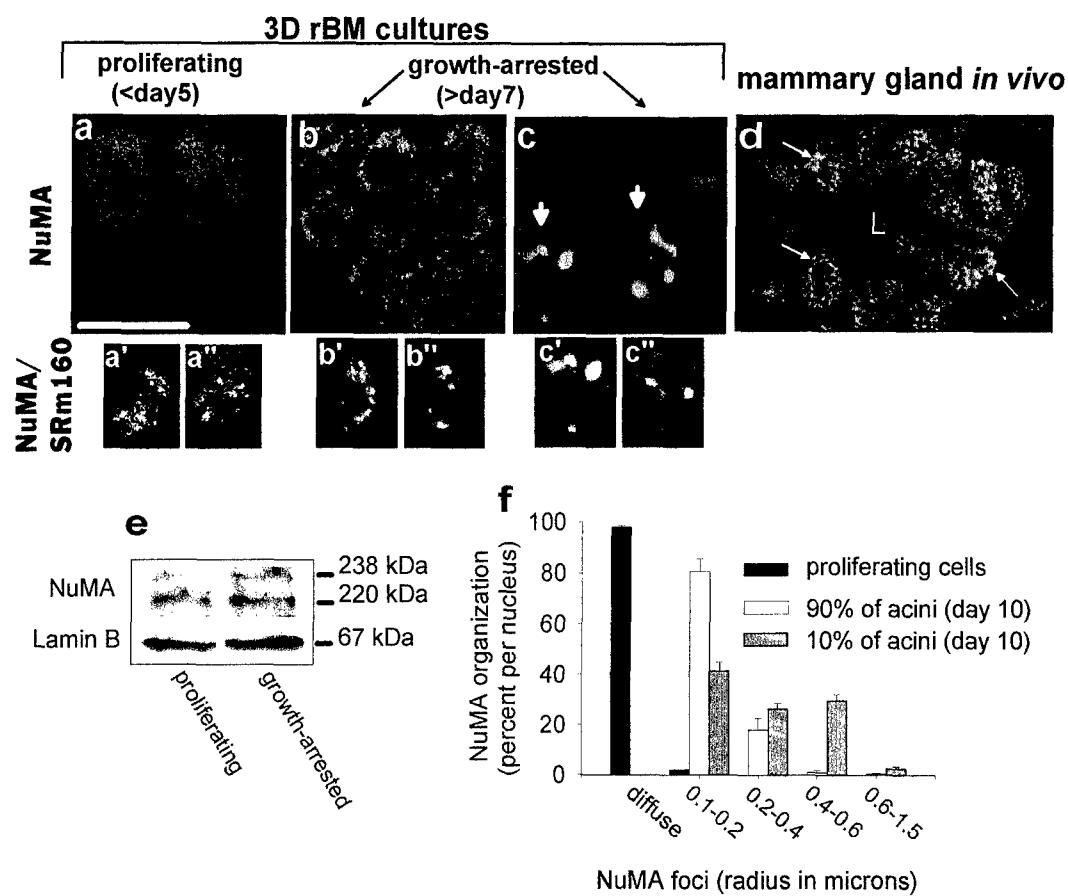
Lamin B   **NuMA**   **SRm160**

monolayer
intact cells

nucleus

acinus
intact cells

nucleus

acinus
in situ NM

nucleus

Figure 1

Figure 2A

Figure 2B
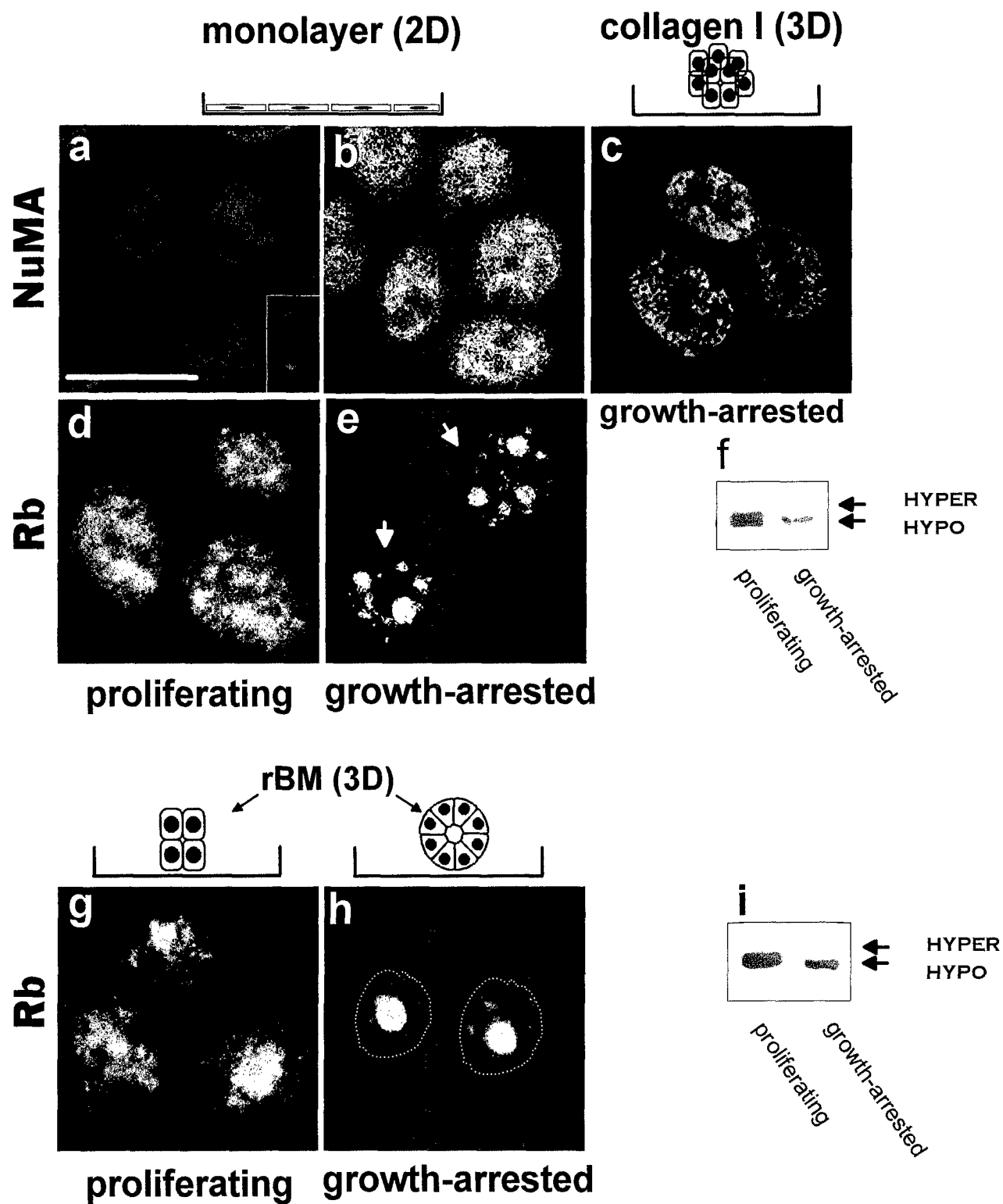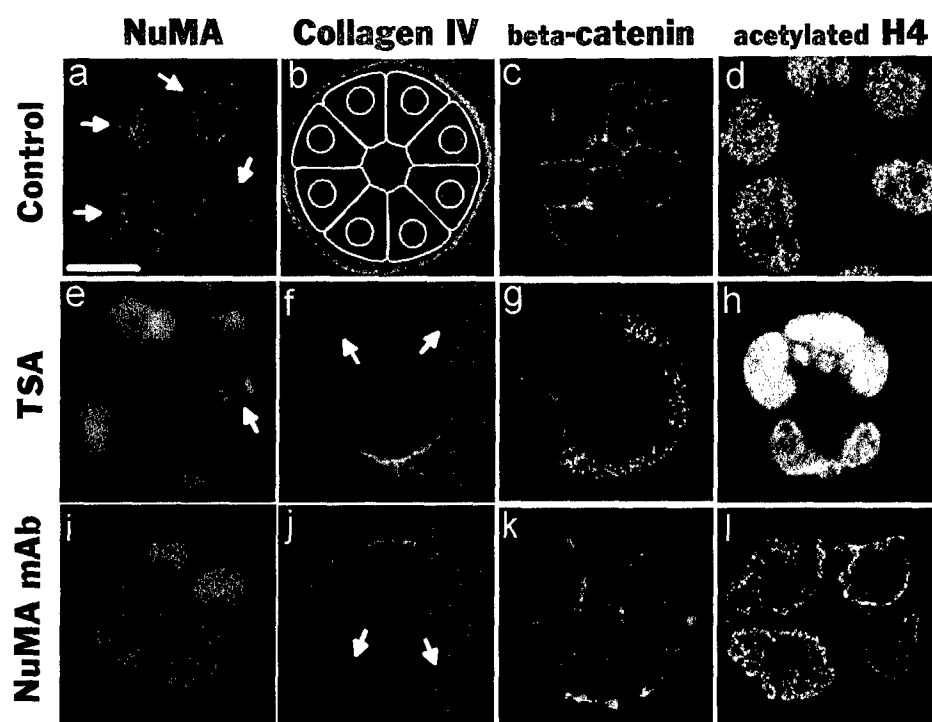
monolayer (2D)   collagen I (3D)

NuMA

a   b   c

growth-arrested

Rb

d   e   f

HYPER
HYPO

proliferating   growth-arrested

proliferating   growth-arrested

rBM (3D)

Rb

g   h   i

HYPER
HYPO

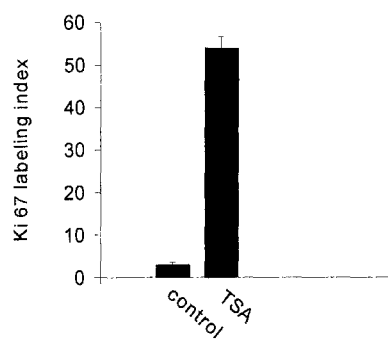proliferating   growth-arrested
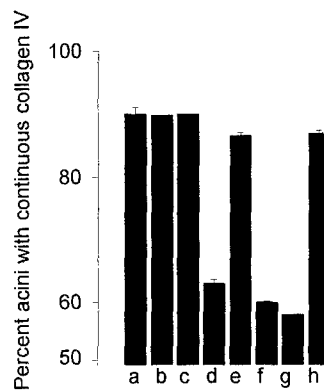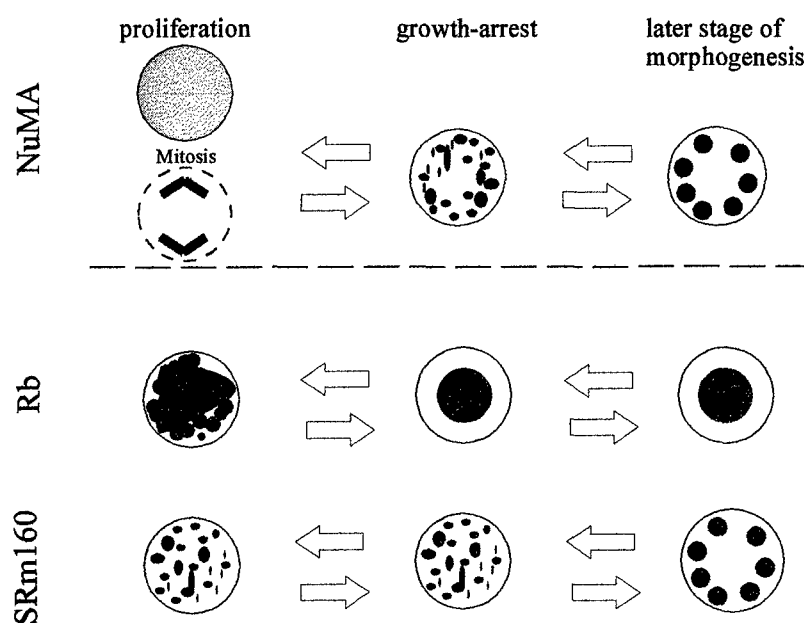
proliferating   growth-arrested

Figure 3

Figure 4

Dynamics of the distribution of NM proteins in 3D rBM

(Only the cell nucleus is represented, NuMA in red; Rb and SRm160 in green)

Scheme 1

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   21 Mar 01

MEMORANDUM FOR Administrator, Defense Technical Information
           Center, ATTN: DTIC-OCA, 8725 John J. Kingman
           Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Changes in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has
reexamined the need for the limitation assigned to technical
reports written for Grant DAMD17-97-1-7103. Request the limited
distribution statements for Accession Document Numbers ADB248428
and ADB262542 be changed to "Approved for public release;
distribution unlimited." These reports should be released to the
National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at
DSN 343-7322 or by email at judy.pawlus@amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
   Information Management